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## **Introduction:**

This research program will determine whether accelerated neuron death due to increased oxidative stress resulting from mitochondrial dysfunction can be compensated or corrected by neurotrophin stimulation. The experiments will be carried out in hippocampal neurons from the trisomy 16 mouse, which undergo increased apoptosis and have a mitochondrial defect, and in neurons chronically treated with the neurotoxin rotenone to induce a defect in mitochondrial function. A unique aspect of this approach is that the neuronal responsiveness to brain derived neurotrophic factor (BDNF) will be enhanced by overexpressing the BDNF receptor via an adenovirus vector, resulting in an increase in sensitivity to BDNF. Such neurons would be expected to have an enhanced survival response to endogenous BDNF and may be more resistant to oxidative stress characteristic of Parkinson's disease and other neurodegenerative disorders.

## **Overview, Year One:**

I have shown a decrease in State 3 respiration in trisomy 16 brain. This is a confirmation of my hypothesis (Aim One) that there is a defect in mitochondrial function in Ts16. A major technical problem experienced this year has been the difficulty of doing oxygen respiration studies with the small amounts of tissue available from fetal trisomic mouse brains, which necessitating pooling 5-8 mouse brains (5-8 pregnant mice) for each experimental point. I have recently overcome this difficulty by identifying a modification of the respiration techniques used in Dr. Fiskum's laboratory which will allow me to measure rates using significantly (up to 10-fold) less tissue. With regard to the goals of Aim Two, I have completed a series of chronic rotenone dose response curves using primary hippocampal neurons and shown that, as expected, they are much more sensitive to the effects of low doses of rotenone than are neuroblastoma cells and that chronic rotenone treatment can leave the neurons more vulnerable to a second oxidative stress.

In this first year of the research project several administrative goals have been reached which will underlie the work of the next three years. These include: setting up the mouse colony required for the trisomy 16 mouse studies; the identification and hiring of a high quality technician (Ms. Weili Dai); and training the technician in hippocampal dissection and neuron culture. Although Ms. Dai did not start working for me until March, I was able to hire a summer student (Ms. Shira Heletz). Having two technicians for the summer compensated for the project not having one earlier in the year and allowed me to make the progress outlined above.

## **Research Accomplishments, Year One, Aim One:**

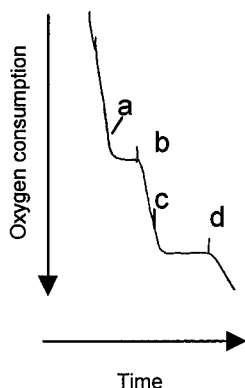


Fig. 1. Respiration in permeabilized astrocytes. Astrocytes 6 days in vitro were suspended at a concentration of  $9 \times 10^6$  cells/ml of KCl medium containing (in mM) 125 KCl, 2  $K_2HPO_4$ , 1  $MgCl_2$ , 20 HEPES (pH 7), 5 glutamate, 5 malate 1 ADP, 37°C and permeabilized with 0.003% digitonin. The additions were: a. 4  $\mu$ M rotenone; b. 5 mM succinate; c. 0.5  $\mu$ M antimycin A; d. FCCP.

It has been suggested that there is a complex I defect in Ts16 mitochondria (Schuchmann and Heinemann, 2000) on the basis of differing effects of the complex I inhibitor, rotenone, on free radical generation in euploid and Ts16 neurons. However, these results are difficult to interpret, since they showed an inhibition of ROS by rotenone, in contrast to the increase more commonly seen when rotenone prevents complex I from passing electrons on down the electron transport chain (ETC)

(see e.g. Sherer et al, 2001; 2002). It is possible that these results reflect artifactual changes in hydroethidine fluorescence. Thus, the question of whether there is an ETC defect in Ts16 remains unanswered. ETC defects in Ts16 could lead to increased oxidative stress and decreased energy

production and predispose Ts16 neurons to die. Understanding how an ETC defect is generated in these mice will improve our understanding of how such defects can occur in human neurodegenerative diseases.

#### Oxygen consumption in Ts16 brain.

Electron transport chain activity can be measured using an oxygen electrode with isolated mitochondria, cells permeabilized with digitonin, or tissue homogenates (Fiskum et al., 2000). An example using

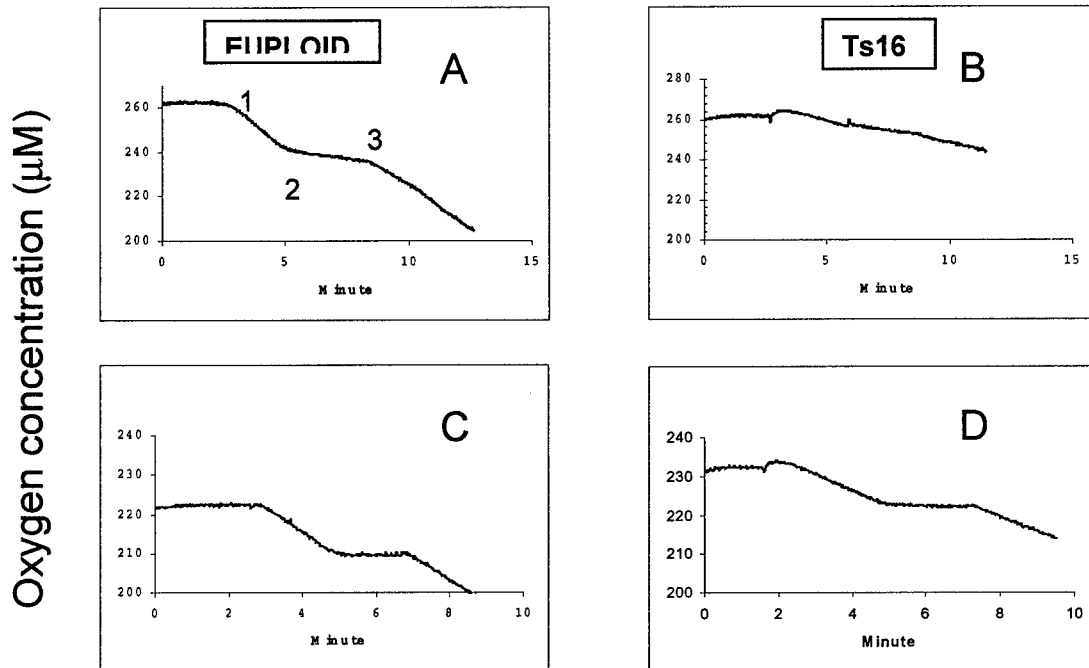


Figure 2. Oxygen respiration with euploid (A,C) and Ts16 (B,D) cortex from two experiments (A&B and C&D). All the experiments were performed as indicated in A: respiration was initiated by addition of homogenate (1), followed by oligomycin (2) and FCCP (3), as in Figure 1.

permeabilized astrocytes is shown in Figure 1. The initial rate of oxygen consumption (state 3) is measured in the presence of the oxidizable complex I substrates glutamate and malate. Respiration stops after addition of the complex I inhibitor rotenone (a) and can be restarted by addition of the complex II substrate succinate (b). The complex III inhibitor antimycin blocks respiration again (c) but it can be restarted with addition of the uncoupler FCCP (d). Neuron yields from E16 hippocampal preparations are typically 400,000 cells/brain. Since on the order of  $10^7$  cells are needed for oxygen consumption measurements in permeabilized cells, we cannot do these experiments in purified hippocampal cultures. Since it is probable that the Ts16 defect is present in all neurons, we have studied respiratory parameters in tissue homogenates (see Kuroda and Siesjo, 1997; Sims and Pulsinelli, 1987) of euploid and Ts16 cortex. Respiration was measured polarographically in KCl medium (Figure 1) at 37°C with a Clarke electrode (Fiskum et al., 2000).

Brain homogenates were prepared as in Kunz et al. (2000) by homogenization of E16 cortex on ice in KCl medium with 0.5 mM EDTA. Protein was determined using the micro-Lowry method. The maximal rate of coupled respiration was determined in the presence of glutamate and malate as in Figure 1 (state 3). Then ATP synthesis was inhibited with oligomycin to measure resting (state 4) respiration, which reflects the rate of leakage of protons back across in inner mitochondrial membrane. Respiration was then restarted with the addition of the uncoupler FCCP, which acts as a proton ionophore to allow protons to flow freely into the mitochondria.

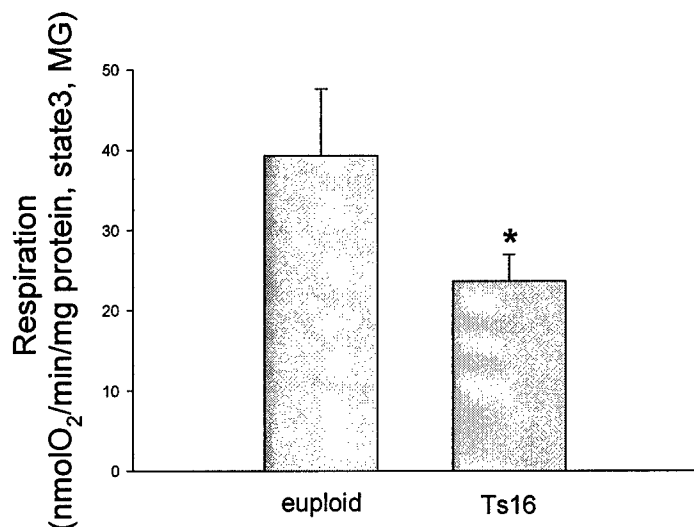


Figure 3. State 3 respiration in the presence of malate and glutamate (MG) in euploid and Ts16 cortices. Data are means  $\pm$  SEM for  $n=5$  experiments, \* significantly different,  $p<0.05$  by  $t$ -test

Figure 2 shows representative results from two experiments comparing respiration using malate/glutamate (complex 1 substrates) as substrates. After the KCl medium containing  $Mg^{2+}$ , malate, glutamate and ADP is mixed; the reaction is initiated by the addition of 20-40  $\mu$ l of brain homogenate in a final volume of 0.5 ml. The Clark oxygen-sensing electrode measures the changing concentration of oxygen in the medium. The addition of oligomycin blocks the ATPase; the remaining oxygen consumption is then driven by proton leak across the mitochondrial membrane. The addition of the proton ionophore FCCP uncouples respiration from ATP production and allows oxygen consumption to resume. The first point

to be drawn from these experiments is that the absolute changes in oxygen concentration are very small, on the order of 10-20  $\mu$ M. This makes the determination of the slopes, from which the rates are calculated, less certain. In the case of the oligomycin-inhibited rate, the absolute changes are so small that meaningful comparisons between euploid and Ts16 brains cannot be made. Figure 2 also shows the variability in oxygen consumption seen between different preparations, this is partially due to changes I have made in isolating the brains to improve yield and respiration. Nevertheless, repeated experiments have consistently found a lower rate of malate/glutamate supported respiration in Ts16, as summarized in Figure 3. This finding is consistent with the hypothesis that there is a defect in Complex I in Ts16.

State 4 rates can increase in the presence of membrane permeability transition pore (MPT) opening, or  $Ca^{2+}$ - or free-radical induced membrane damage. Increased State 4 respiration in Ts16 would be consistent with  $Ca^{2+}$ - or oxidative stress-induced mitochondrial damage. If the ratio of State 3/State 4 (the respiratory control ratio, RCR). If the RCR is significantly lower in Ts16 as compared to euploid brains, this would reflect a greater proton leak in Ts16. However, the very small absolute

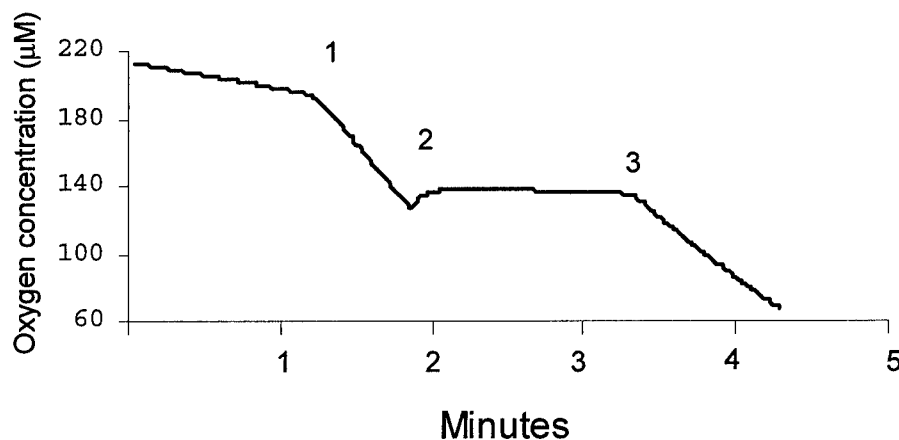


Figure 4. Respiration for euploid cortex measured in the StrathKelvin Minicell. Cortex was mixed with malate, glutatmate,  $Mg^{2+}$  in KCl buffer, respiration was initiated by the addition of ADP (1), blocked by oligomycin (2) and restarted with FCCP (3).

changes in oxygen concentration made a reliable determination of state 4 respiration difficult. Thus, although the Ts16 brain mitochondria do appear less well coupled than euploid (Figure 2), this important point has not yet been quantitatively confirmed, due to the difficulty of accurately measuring respiration in the presence of oligomycin.

In order to deal with the technical difficulty of accurately measuring small changes in oxygen consumption, I have recently tested and purchased a new oxygen electrode setup from Strathkelvin laboratories. With this setup, I can measure oxygen consumption in a volume as low as 50  $\mu$ l. In this procedure, 2-5 cortices are homogenized in ice-cold KCl buffer with EDTA, centrifuged once at 4,000rpm for 4 minutes and then again at 10,000rpm for 15 minutes. The resulting pellet is resuspended in 50-100  $\mu$ l of KCl buffer and 10  $\mu$ l is used/assay. The brain suspension, substrates and  $Mg^{2+}$  are mixed, the chamber is sealed and the reaction initiated by the addition of ADP. Figure 4 shows that using the Strathkelvin Minicell, absolute changes in oxygen consumption on the order of 100  $\mu$ M in 1 minute are obtained. This is the method that will be used in subsequent experiments and I am confident that it will allow for the detailed characterization of the mitochondrial defect revealed by the experiments in Figure 3.

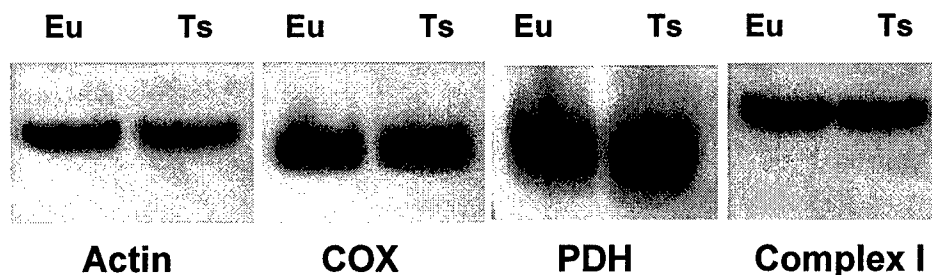


Figure 5. Representative Western blots for 25  $\mu$ g of euploid and Ts16 cortex probed for actin, COX, PDH or Complex 1 as described in the text. Similar results were obtained in three separate experiments.

A possibility that arose during these experiments was that Ts16 brain might have a lower mitochondrial mass (fewer or smaller mitochondria) per unit of total protein than euploid brain. I therefore performed Western Blot analysis of the brain homogenates used in the respiration studies for pyruvate dehydrogenase (PDH), cytochrome oxidase (COX, Complex IV, subunit I) and Complex I (NADH-ubiquinone oxidoreductase I, subunit  $\alpha$ ) (Antibodies from Molecular Probes). The blots were stripped and reprobbed for actin to control for loading differences. Representative blots are shown in Figure 5. There was no apparent difference in the level of expression of these mitochondrial proteins between Ts16 and euploid brain. Therefore, the observed difference in respiration is likely to reflect a change in the function of the mitochondria in the trisomic mice.

### **Research Accomplishments, Year One, Aim Two:**

#### **In vitro model of oxidative stress in primary CNS neurons: chronic rotenone treatment of euploid hippocampal neurons.**

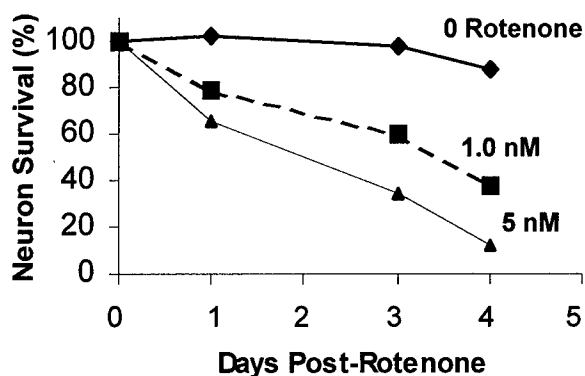


Figure 6. Hippocampal neuron survival in the presence of 0, 1, 5 nM rotenone added on day 2 in vitro. Survival is expressed as a percentage of the cells present when the rotenone was added, Data are the means of 6 fields on two coverslips.

Chronic (2-4 wks) treatment with 5 nM rotenone has been shown to render cultured neuroblastoma cells more vulnerable to degeneration induced by oxidative stress ( $H_2O_2$ ) and more likely undergo abnormal repetitive  $Ca^{2+}$  spiking subsequent to stimulation (Sherer et al., 2001; 2002). The effect of this treatment has not been studied in primary cultured neurons, which may respond differently than do cell lines such as neuroblastoma cells, which are transformed, proliferating cell lines that do not have all of the characteristics of CNS neurons. We have

measured the survival of euploid hippocampal neurons in the absence and presence of  $H_2O_2$  following chronic rotenone treatment.

Euploid hippocampal cultures maintained in serum-free medium supplemented with B27 were treated with rotenone at 2 div and neuron survival was monitored as described (Bambrick et al., 1995; Bambrick and Krueger, 1999). In the initial experiments (Figure 6) both 5 nM (the dose used with neuroblastoma cells- Sherer et al., 2002) and 1 nM rotenone led to over 70% cell death after only 4 days. Thus the previously reported conditions used for neuroblastoma cells are not appropriate for primary neurons. Primary hippocampal neurons appear to be much more sensitive to oxidative stress. This may reflect adaptations that have occurred during the transformation and propagation of the neuroblastoma cell line and support the importance of using primary cells in studies of neuron death.

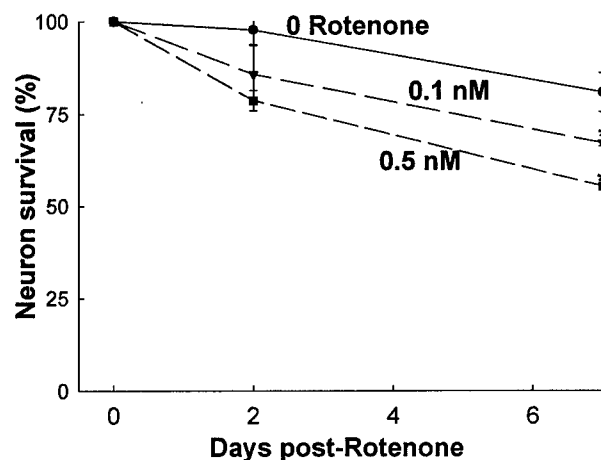


Figure 7. Hippocampal neuron survival in the presence of 0, 0.1, 0.5 nM rotenone added on day 2 in vitro. Survival is expressed as a percentage of the cells present when the rotenone was added. Data are the means and SEM for 4 separate experiments. \* significant difference from control as determined by ANOVA.

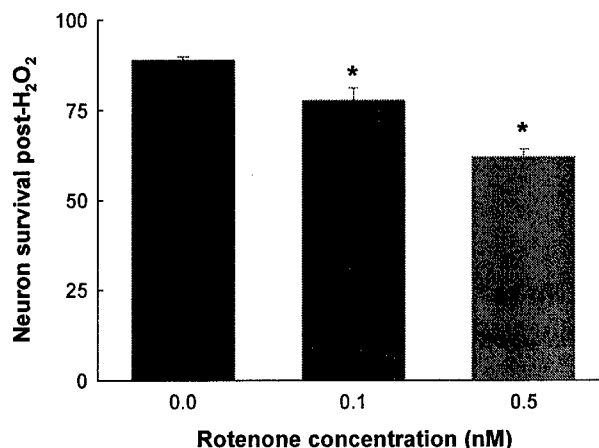


Figure 8. Hippocampal neurons were treated with rotenone for 7 days as in Figure 7. The cultures were then treated with 10  $\mu M$   $H_2O_2$  for 24 hours. Survival is the percentage of  $H_2O_2$ -treated versus untreated cultures. Data are means and SEM for 3 experiments, \* is significantly different from control at  $p < 0.05$ .

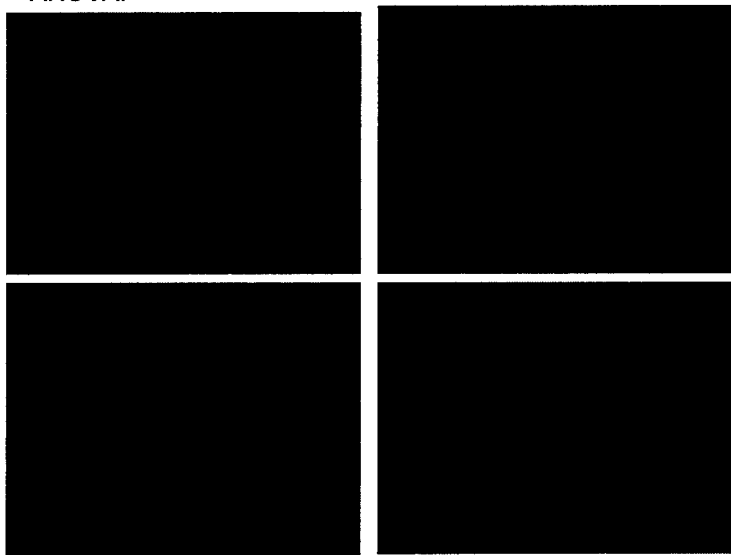


Figure 9. Immunocytochemistry for Nitrotyrosine (green) in hippocampal cultures treated for 6 days with 0 (top) or 5 nM (bottom) rotenone. The same fields are shown counterstained with DAPI (blue) to show the nuclei.

In subsequent experiments I have now determined that treatment with 0.1 and 0.5 nM rotenone allows over 50% of the treated hippocampal neurons to survive for at least one week (Figure 7). By this time there is a significant increase in the sensitivity of the rotenone treated cells to a second oxidative stress (10  $\mu M$   $H_2O_2$ ) (Figure 8). Immunocytochemistry shows increased levels of nitrotyrosine residues, which are formed in the presence of increased NO radicals, after one week in 0.5 nM rotenone (Figure 9), indicating increased oxidative stress in these cells. Future experiments will use a ELISA assay to quantify levels of nitrotyrosine.

### **Key Research Accomplishments**

- Mitochondria from Ts16 brain show a significant inhibition of Complex I-mediated respiration that is not due to a decrease in mitochondrial mass.
- Treatment of cultured hippocampal neurons with low doses (0.1-0.5 nM) rotenone over one week gives rise to cells with increased levels of nitrotyrosine, a marker of oxidative stress, and an increased susceptibility to subsequent treatment with H<sub>2</sub>O<sub>2</sub>.

### **Conclusions**

The first year of this four year grant has laid the groundwork for the studies to follow. The Ts16 mouse colony has been established. A technician has been hired and trained and she is now proficient in the hippocampal neuron cultures.

There has been significant progress made on both the Aims of the initial grant proposal. The hypothesis that there is a defect in Ts16 metabolism has been confirmed and shown to be a measurable decrease in Complex I-mediated respiration. The technical difficulties involved in measuring respiration in small amounts of fetal brain tissue has been overcome by the identification and purchase of a new oxygen electrode and chamber- made possible by this grant. This direct proof of a mitochondrial defect in Ts16 is important because I will now be able to determine where in the electron transport chain this defect occurs, whether it leads to an increased generation of free radicals and whether this can be ameliorated by neurotrophin therapy. With regard to the last point, our earlier work (Dorsey et al., 2002) had shown that Ts16 neurons have defective BDNF signaling because of an over expression of the truncated form of the BDNF receptor trkB (which acts as a dominant negative repressor). Dr. Susan Dorsey has now developed a truncated trkB knockout mouse (see Appendix I). Using this mouse I will generate Ts16/truncated trkB-negative mice. This will allow me to directly test the hypothesis that Ts16 mice have a mitochondrial defect that is separate from their defective trkB signaling. If my preliminary experiments generating these mice and measuring mitochondrial respiration are successful, I will then apply to amend the original SOW to take advantage of this newly available resource.

The experiments directed towards establishing a chemical model of chronic mitochondria dysfunction have also been successful. Primary neurons were found to be much more sensitive to oxidative stress than neuroblastoma cells. Dose response curves with chronic rotenone treatment showed that a low dose of rotenone that allowed the survival of up to 70% of the neurons left the surviving cells susceptible to a second oxidative stress. This model will now be used to test the ability of neurotrophin therapy to compensate for mitochondrial dysfunction.

## References

- Bambrick LL, Yarowsky PJ, Krueger BK (1995) Glutamate as a hippocampal neuron survival factor: an inherited defect in the trisomy 16 mouse. *Proc.Natl.Acad.Sci.USA* 92:9692-9696.
- Bambrick LL, Krueger BK (1999) Neuronal apoptosis in mouse trisomy 16: mediation by caspases. *J.Neurochem.* 72:1769-1772.
- Dorsey SG, Bambrick LL, Balice-Gordon RJ, Krueger BK. (2002) Failure of brain-derived neurotrophic factor-dependent neuron survival in mouse trisomy 16. *J.Neurosci.* 22:2571-2578.
- Fiskum G, Kowaltowski AJ, Andreyev AY, Kushnareva YE, Starkov AA (2000) Apoptosis-related activities measured with isolated mitochondria and digitonin-permeabilized cells. *Meth.Enzymol.* 322:222-234.
- Kunz J, Schneider U, Howald I, Schmidt A, Hall MN (2000) HEAT repeats mediate plasma membrane localization of Tor2p in yeast. *J Biol Chem* 275: 37011-37020.
- Kuroda S, Siesjo BK (1997) Reperfusion damage following focal ischemia: pathophysiology and therapeutic windows. *Clin.Neurosci.* 4:199-212.
- Schuchmann S, Heinemann U (2000) Increased mitochondrial superoxide generation in neurons from trisomy 16 mice: A model of Down's syndrome. *Free Radical Biol.Med.* 28:235-250.
- Sherer TB, Betarbet R, Greenamyre JT. (2002) Environment, mitochondria, and Parkinson's disease. *Neuroscientist.* 8:192-7.
- Sherer TB, Betarbet R, Stout AK, Lund S, Baptista M, Panov AV, Cookson MR, Greenamyre JT (2002) An *in vitro* model of Parkinson's disease: linking mitochondrial impairment to altered  $\alpha$ -synuclein metabolism and oxidative damage. *J.Neurosci.* 22:7006-7015.
- Sherer TB, Trimmer PA, Borland K, Parks JK, Bennett JP, Jr., Tuttle JB (2001) Chronic reduction in complex I function alters calcium signaling in SH-SY5Y neuroblastoma cells. *Brain Res.* 891:94-105.
- Sims NR, Pulsinelli WA (1987) Altered mitochondrial respiration in selectively vulnerable brain subregions following transient forebrain ischemia in the rat. *J.Neurochem.* 49:1367-1374.

## Appendix One

ABSTRACT: Dorsey, S.G. and Tessarollo, L. (2003). In Vivo Analysis of Truncated trkB.T1 Function Using a Conditional Gene Targeting Approach. Gordon Research Conference on Neurotrophic Factors. June 8-13, Salve Regina University, Newport, RI.

There are four receptor isoforms encoded by the trkB gene; a full-length, catalytically active isoform, a trkB-T-Shc isoform, and two alternatively-spliced truncated isoforms, T1 and T2, which lack kinase activity but have unique intracellular domains of nine and eleven amino acids respectively. All four isoforms share identical extracellular and transmembrane domains, and are capable of binding BDNF with equal affinity. TrkB.T2 is expressed at extremely low levels in human, mouse, and rat. TrkB.T1, the predominant truncated isoform, is expressed in the mammalian CNS, PNS, and non-neuronal tissues such as lung, spleen, testis, kidney, intestine and heart.

The role of truncated trkB has not been fully elucidated, however, several observations argue for its' importance. For example, it is dynamically up-regulated during later stages of fetal development, and becomes the predominant trkB receptor isoform in adulthood. In some neural cell types, such as astrocytes, the truncated trkB is the only receptor isoform expressed. Moreover, this receptor is conserved across species, and throughout evolution. From *in vitro* and *in vivo* data, several roles for truncated trkB have been proposed, including dominant negative inhibition of trkB-mediated signal transduction by heterodimerizing with the full-length isoform, ligand sequestration, or independent signal transduction through as yet unidentified pathways. The purpose of this project is to investigate the function of truncated trkB.T1 *in vivo* using a conditional gene-targeting approach.

The mouse trkB locus is approximately 326 kb and contains 17 exons. The T1 exon is located between exons 11 and 12, and is flanked by approximately 50 kb of intronic sequence. The knockout construct, engineered using a recombinogenic cloning strategy, was designed to target the T1 exon without disrupting transcription and translation of the full-length trkB receptor. Having recently obtained germline transmission of the trkBT1-targeted allele, we plan to breed heterozygous animals to homozygous  $\beta$ -actin CRE mice to obtain T1 knockout animals for analysis, the results of which will be presented.